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# Inflammatory responses following intramuscular and subcutaneous immunization with aluminum-adjuvanted or non-adjuvanted vaccines

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ABSTRACT

Aluminum-adjuvanted vaccines are administered through an intramuscular injection (IM) in the US and EU, however, a subcutaneous injection (SC) has been recommended in Japan because of serious muscle contracture previously reported following multiple IMs of antibiotics. Newly introduced adjuvanted vaccines, such as the human papillomavirus (HPV) vaccines, have been recommended through IM. In the present study, currently available vaccines were evaluated through IM in mice. Aluminum-adjuvanted vaccines induced inflammatory nodules at the injection site, which expanded into the intra-muscular space without any muscle degeneration or necrosis, whereas non-adjuvanted vaccines did not. These nodules consisted of polymorph nuclear neutrophils with some eosinophils within the initial 48 h, then monocytes/macrophages 1 month later. Inflammatory nodules were observed 6 months after IM, had decreased in size, and were absorbed 12 months after IM, which was earlier than that after SC. Cytokine production was examined in the injected muscular tissues and AS04 adjuvanted HPV induced higher IL-1β, IL-6, KC, MIP-1, and G-CSF levels in muscle tissues than any other vaccine, but similar serum cytokine profiles were observed to those induced by the other vaccines. Currently available vaccines did not induce muscular degeneration or fibrotic scar as observed with muscle contracture caused by multiple IMs of antibiotics in the past.



## 1. Introduction

All vaccines have been administered through a subcutaneous injection (SC) in Japan, whereas aluminum-adjuvanted vaccines are administered through an intramuscular injection (IM) without any serious reactions in the EU, US, and many other countries [1]. IM was prohibited in Japan because serious muscle contracture was reported with multiple IMs of antibiotics with or without antipyretics in the 1960s. The first case of the muscle contracture was reported by an orthopedic surgeon in 1947, and may have been caused by IM of antibiotics. The number of these cases increased and several regional accumulations of patients were reported, especially in Yamanashi prefecture, where legal action was taken. All cases had multiple IMs of antibiotics with or without antipyretics, but not with vaccines. The Japanese Orthopedic Association announced the Precaution in 1976 that muscle contracture was mainly caused by IM of antibiotics and that pediatricians should refrain from unnecessary IM. Thereafter, an Investigational Committee on Muscle Contracture was established by the Japanese Pediatric Association, which announced the following comments in 1977 [2]:

1) Muscle contracture was reported in the quadriceps, deltoids, and buttocks, and no site was safe for IM.
2) Muscle contracture was reported in all age groups, not just in young infants.
3) The indication of IM was extremely rare.
4) Informed consent had to be obtained from patients or their guardian in cases in which IM was required.

The histopathological findings obtained from the muscle tissues of the patients revealed the infiltration of inflammatory cells, degeneration of muscle cells, necrosis, fibrosis, and scar formation, which were similar to those observed in experimental animals following IM of various antibiotics [3–5]. IM was

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subsequently prohibited for all medicinal procedures except the administration of immunoglobulin preparations. All vaccines were administered through SC, and the Committee on Muscle Contracture also suggested that all medicinal preparations for IM had to be histopathologically examined in the muscle tissues of experimental animals to assess the damage to muscle tissue [2].

Serious local reactions were previously reported following immunization with diphtheria and tetanus toxoids combined with the acellular pertussis vaccine (DPT) containing an aluminum adjuvant, and the precise mechanisms underlying local reactogenicity and immunogenicity have not fully elucidated [6–8]. In addition to a conventional aluminum adjuvant, a new vaccine containing monophosphoryl lipid A (MPL) was introduced [9]. Aluminum has been used as an adjuvant for a long time because it prolongs the retention of adsorbed antigens at the injection site (depot effect), however, recent findings on innate immunity have indicated that aluminum adjuvants initiate primary immune-stimulation in the innate immune system [10,11]. Innate immunity consists of two different patterns: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). All effective vaccines stimulate the innate immune system to produce cytokines or chemokines for the development of acquired immune responses through the expression of co-stimulatory molecules [12–14]. These reactions start in the early phase following the injection: therefore an investigation of local reactions following a vaccination appears to be warranted to better understand the safety and immunogenicity of vaccines [12,15,16].

*Haemophilus influenzae* type b (Hib) was introduced in Japan in 2008, 7-valent pneumococcal (PCV7) and human papillomavirus (HPV) vaccines in 2010 [17]. These newly introduced vaccines are administered through IM in other countries. However, only HPV vaccines are recommended through IM, as is stated on the package inserts. Vaccination against HPV has been associated with a chronic pain syndrome in Japan, although a causal relationship has not been established [18]. All routine vaccines, including newly introduced ones, have not been examined to assess the safety of IM administration: therefore, histopathological findings and local cytokine production were investigated in the present study using current available inactivated vaccines.

## 2. Materials and methods

### 2.1. Vaccines

All routine inactivated vaccines were examined. DPT (Kitasato Institute, Japan), Hib (Sanofi Pasteur, France), PCV7 (Pfizer, USA), the Japanese Encephalitis vaccine (JEV) (Biken, Japan), seasonal influenza split vaccine (Kitasato Institute, Japan), 4-valent HPV (Gardasil: MSD, USA), and 2-valent HPV (Cervarix: GSK, Belgium) were purchased commercially.

### 2.2. Experimental design

Four-week-old BALB/c mice were purchased from Charles River, US. All vaccines were administered in 100 μl volumes through IM in the left quadriceps muscle in four mice for each vaccine (1/5 volume of human dose) and phosphate-buffered saline (PBS) at the right quadriceps muscle for the control. Muscle tissues were examined 1 month after a single injection to compare histological findings by different vaccine preparations. Mice were immunized with three doses of DPT through IM in the same left quadriceps, or through SC in the back of the neck, to compare pathological findings through IM and SC. Injection sites were examined 1, 3, 6, 9, and 12 months after the injection to assess local reactions. Sera were also obtained to compare serological responses.

To assess cytokine responses and histological findings at very early phase following the injection, quadriceps muscle tissues and serum samples were obtained pre, 3, 6, 24, and 48 h after a single injection of DPT, Hib, PCV7, JEV, Cervarix, and Gardasil in three mice for each point. PBS was injected in the opposite quadriceps as the control.

### 2.3. Histological examinations

Quadriceps muscle tissues were fixed with 10% phosphate-buffered formalin and decalcified in PBS before embedding in paraffin. Muscle and subcutaneous tissues were stained with hematoxylin and eosin (HE) using a conventional procedure. Lumogallion staining was performed and aluminum compounds were visualized through confocal microscopy [19]. Macrophages were stained with antibodies against F4/80 (a rat monoclonal antibody against mouse F4/80, AbD Serotec, USA), iNOS (polyclonal rabbit anti-iNOS/NOS type II, BD, USA), and arginase I (rabbit polyclonal antibody against human arginase, Santa Cruz, USA) [20–22].

### 2.4. Cytokine productions

Quadriceps muscles were harvested, cut into small pieces, and homogenized with 2 ml of RPMI supplemented with 1% protease inhibitor (nacalai tesque, Kyoto, Japan) using Bio Masher II (Nippi, Tokyo, Japan). The muscle homogenate was centrifuged, filtrated through a 0.45 μm filter, and subjected to a cytokine assay. IL-1β, IL-2, IL-4, IL-6, IL-10, Eotaxin, G-CSF, KC, MCP-1, and TNF-α were measured using the BioPlex mouse cytokine panel (BioPlex, Bio-Rad Laboratories, USA). The local production of cytokines was expressed as the ratio of the cytokine concentration at the injected site to that at the opposite site injected with PBS, and the mean of three mice was shown for each cytokine.

### 2.5. Statistical analyses

Differences between the groups were analyzed using Cochran–Cox method and a significant difference was defined as $p < 0.05$, using StatMate software (ATMS, Tokyo).

## 3. Results

### 3.1. Histological findings 1 month after the single dose injection

Hib, influenza, and JEV do not contain aluminum adjuvant. The DPT vaccine consists of 300 μg/ml of aluminum, PCV 250 μg/ml, Gardasil 450 μg/ml, and Cervarix contains 1.0 mg/ml together with 100 μg/ml of monophosphoryl lipid A (MPL) adjuvant. Histological findings following IM immunization are shown in Fig. 1. Histopathological findings differed in muscle tissues injected with aluminum-adjuvanted or non-adjuvanted vaccines. No significant difference was observed in the pathological findings obtained from tissues injected with non-adjuvanted JEV vaccine. However, one of the three mice injected with Hib exhibited small localized focal inflammatory reactions with the infiltration of inflammatory and myogenic cells. Similar findings were observed in one of the four mice immunized with the influenza vaccine. Non-adjuvanted vaccines induced no significant pathological differences or small localized inflammatory reactions.

Aluminum-adjuvanted vaccines induced inflammatory nodules with the infiltration of inflammatory cells or macrophages at the marginal lesions. Inflammatory nodules spread into muscle bundle spaces without the degeneration of or atrophic changes to muscle cells. Infiltrating cells were characterized as macrophages: ballooned cytoplasm with peripherally localized nucleus. Lumogallion staining was performed to visualize the aluminum adjuvant



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